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EXPERIMENTAL STUDY

The involvement of apoptotic regulators during *in vitro* decidualization

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Abstract

Objectives: The uterus responds to an implanting blastocyst by undergoing extensive tissue modification leading to decidualization. This modification includes differentiation and apoptosis of epithelial as well as stromal cell compartments. It is generally accepted that the decidual cell regression pattern is similar to the pattern of initial differentiation, suggesting that decidual cell death is the end point of timed differentiation. However, the molecular mechanisms controlling these events are not understood clearly. Therefore, we aimed to investigate the involvement of apoptotic factors using an *in vitro* cell culture system.

Design: In order to assess the role of apoptotic factors during decidualization, we used a decidual cell line (GG-AD) that had been transformed with a temperature-sensitive SV-40 mutant. At the non-permissive temperature (39 °C), these cells showed the characteristics of differentiated decidual cells. They dedifferentiated into stromal cells when the temperature was shifted back to 33 °C.

Methods: We performed Northern blot analysis for *bax*, *bcl-x*$_L$ and *bcl-2* at both temperatures. The onset of apoptosis was examined by Annexin V staining. The expression of p53 protein was also determined by Western blot.

Results: We found an increase in the expression of *bax* when GG-AD cells were grown at 39 °C. We also showed apoptosis with Annexin V staining at 39 °C. The p53 protein expression was also similar to that of the animal models, suggesting that the programmed cell death of the decidual cells occurred in a p53-independent manner.

Conclusions: These data indicate that a parallelism exists between the increased expression of pro-apoptotic genes and decidual cell death, similar to animal models. Therefore, an *in vitro* model of GG-AD cells can be used to assess directly the relationship between apoptotic regulators and decidualization and could be used to study the mechanism of decidual cell regression.

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Introduction

During placentation, a well-orchestrated plan that includes cell differentiation, proliferation and apoptosis enables remodeling of the uterus (1). Adhesion of the blastocyst results in the differentiation of the endometrial stroma into decidual cells, which is an important component of the implantation process. The morphological changes that occur during decidualization *in vivo* have been described extensively (2). The majority of these decidual cells regress in a time-dependent manner, and their removal is important because they provide space for the growth of the embryo (3).

After inducing decidualization either by blastocyst implantation or by intracervical mineral oil injection in the ovariectomized rat model, it has been shown that this regression occurs via apoptosis (3, 4). However, molecular mechanisms controlling decidual cell death are poorly understood. Using ovariectomized rat, we previously demonstrated that during decidualization, the expression pattern within the *bcl-2* gene family is altered favoring decidual cell apoptosis (5). Bcl-2 family proteins are composed of both pro-apoptotic and anti-apoptotic members and can homo- or heterodimerize and function in a distal apoptotic pathway common to all multicellular organisms. The ratio of death antagonists (Bcl-2, Bcl-x_L) to agonists (Bax) has been shown to have a critical role in determining the fate of the cells. (6, 7). Recently it has been shown that prolactin (PRL) plays an important anti-apoptotic role in the decidua of the pseudopregnant rats and the disappearance of PRL-receptor (PRL-R) allows cell

death and reorganization of the decidua (8). Thus, precise knowledge of Bcl-2 family gene expression is necessary to understand the mechanism of decidual cell death.

Although animal models have provided useful information regarding the remodeling of the uterus during blastocyst implantation, the difficulty of carrying out perturbations makes it virtually impossible to define molecular mechanisms. Srivastava *et al.* have established a temperature-sensitive cell line (GG-AD) from endocrine cells of rat decidua (9). These cells were isolated from the antimesometrial region of rat uterus and were stably transfected with simian virus temperature-sensitive (SV-40 tsA209) mutant, which harbors a temperature-sensitive mutation required for the maintenance of cell transformation. They show temperature-dependent changes in growth and morphology. At the permissive temperature of 33 °C, they grow unrestricted and have the characteristics of stromal cells. On the other hand, at the non-permissive temperature (39 °C), they differentiate and resemble antimesometrial decidual cells as evidenced by the expression of marker genes such as progesterone receptor, PRL-like protein B, activin-βA, and desmin (9).

Furthermore, it has been shown that when GG-AD cells are maintained at 39 °C the expression of estrogen receptor β (ERβ) (10), interleukin-6 (IL-6), IL-6 receptor and 130 kDa glycoprotein (gp130) (11), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (12) are increased. In the present study, we utilized the GG-AD cell line to assess directly the relationship between apoptotic regulators and decidualization. We hypothesized that the balance within the *bcl-2* family of genes is altered, and increased expression of pro-apoptotic genes in GG-AD cells at the non-permissive temperature of 39 °C would parallel the onset of decidual cell death. We observed that a parallelism exists between the increased expression of pro-apoptotic genes and decidual cell death.

Materials and methods

Cell culture

GG-AD cells were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 1X glutamine (Gibco BRL), 2X antibiotic–antimycotic solution (Gibco BRL), 1X non-essential amino acids (Gibco BRL), 1X sodium pyruvate (Gibco BRL), 0.5% D-glucose (Gibco BRL) and 10% fetal bovine serum (Gibco BRL). Cells were cultured either at 33 °C or 39 °C with 5% CO_2. The medium was changed every third day. For the cell number, 10^5 cells were plated in a 100 mm tissue culture dish and cultured at 33 °C and 39 °C. Cells were trypsinized and counted at days 4, 7, 12, 17 and 21 ($n = 3$) using a hemocytometer.

Total RNA isolation and Northern blot analysis

Total RNA from GG-AD cells was isolated 5 days after starting the culture using RNeasy Mini kit (Qiagen, Santa Clarita, CA, USA) after homogenization with a QIAshredder (Qiagen, Valencia, CA, USA). The concentration of total RNA was determined by measuring the optical density at 260 nm. The concentrations and the quality of the RNA preparations were confirmed by comparing 28S and 18S rRNA bands stained with ethidium bromide on a 1% agarose–formaldehyde gel. The Northern blot procedure utilized in these experiments has been described previously by Nephew *et al.* (13). Each Northern blot analysis was repeated three times. Briefly, 30 μg samples of total RNA were electrophoresed on agarose–formaldehyde gels and then transferred to Duralon (Stratagene, La Jolla, CA, USA) by capillary transfer. Molecular weight marker RNA was run on each gel. Membranes were pre-hybridized for 15 min at 65 °C with Rapid Hybridization Buffer solution (Amersham Life Science, Arlington Heights, IL, USA) and hybridized for 2 h at 65 °C in the same solution with 1.5×10^6 c.p.m./ml ^{32}P-labeled *bax*, *bcl-2*, *bcl-x*$_L$ and *desmin* cDNA probes. After appropriate washings, the membranes were exposed to Kodak XAR X-ray film at − 70 °C for 5 days. Densitometric analysis was performed using the Multi-Analyst software. rRNA values were used to correct intensities of mRNA expression.

Detection of apoptosis with Annexin V

GG-AD cells were grown on coverslips at 33 °C and 39 °C for 5 days. The Apo-Alert Annexin Apoptosis Kit (Clontech, Palo Alto, CA, USA) was used to stain cells with FITC-conjugated Annexin V. Cells treated with 5% ethanol were used as a positive control. The staining of GG-AD cells with FITC-conjugated Annexin was examined by fluorescence microscopy (Nikon).

Detection of p53 levels

For detection of p53 expression, GG-AD cells were grown at 33 °C and 39 °C for 5 days, and cell lysates were prepared by adding 0.6 ml of RIPA buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS with 1 mmol/l phenylmethylsulfonyl fluoride (PMSF); 1 mg/ml aprotinin, leupeptin, pepstatin; 1 mmol/l Na_3VO_4; 1 mmol/l NaF) and 15 μg of protein were loaded per lane on a minigel electrophoresis apparatus. A 7.5% gel was used for p53 detection. After electrophoresis and transblotting of the proteins onto nitrocellulose membranes, the membranes were washed, blocked with 10% non-fat dry milk in 0.2% Tween 20 in PBS, and then incubated with p53 monoclonal antibody DO1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:150 overnight at 4 °C,

followed by incubation with anti-mouse horseradish peroxidase-conjugated IgG at a dilution of 1:15 000 for 2 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence. As a positive control, we used u.v.-irradiated normal human melanocytes, which are positive for p53 expression (14).

Results

The expression of apoptotic regulators during the temperature-induced decidualization of GG-AD cells

At the permissive temperature (33 °C), GG-AD cells show the characteristics of stromal cells and at the non-permissive temperature (39 °C) they differentiate into decidual cells. Since we have shown that expression of *bcl-2* family genes is altered during early pregnancy in our animal model (5), we decided to examine whether these genes were involved during *in vitro* decidualization. RNA was isolated from cells grown at the permissive temperature (33 °C) or at the non-permissive temperature (39 °C) and *bax*, *bcl-*x_L and *bcl-2* expression were analyzed using Northern blot analysis. As shown in Fig. 1A, *bax* mRNA showed a remarkable induction at 39 °C. The expression of *bcl-*x_L (Fig. 1B) also seems to be induced but the level of increase was not as dramatic as that of *bax*. The expression of *bcl-2* was neither detectable at 33 °C nor at 39 °C (Fig. 1C). Since *desmin* is a widely accepted decidual cell marker (15), we used *desmin* expression to monitor the temperature-induced decidualization of GG-AD cells. The expression of *desmin* shown in Fig. 1D confirmed that GG-AD cells decidualized at 39 °C. Relative expression of these genes was quantitated and normalized using levels of rRNA. The changes in the expression of *bax*, *bcl-*x_L and *desmin* at 33 °C and 39 °C are shown in Fig. 1F. Thus, this cell line seems to mimic what we have observed during the decidualization of stromal cells *in vivo*.

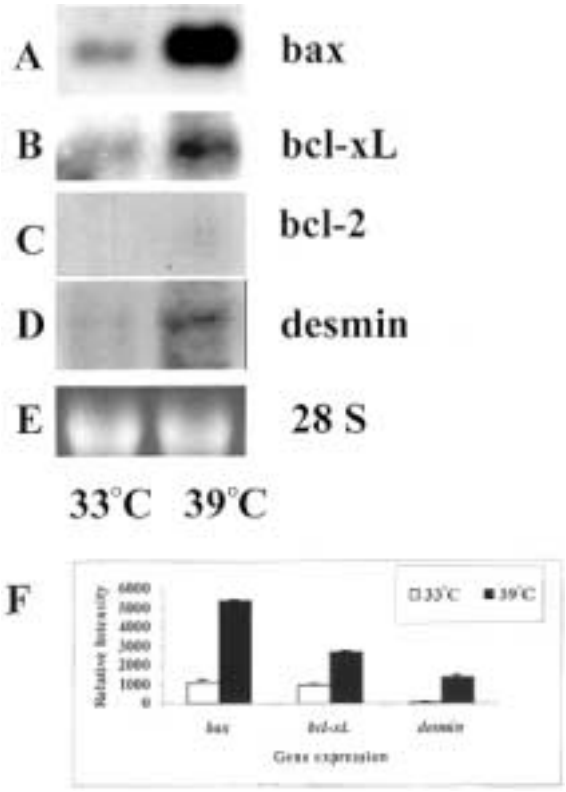

Figure 1 The expression of *bax* (A), *bcl-*x_L (B), *bcl-2* (C) and *desmin* (D) during temperature-induced decidualization of GG-AD cells. GG-AD cells show characteristics of stromal cells at 33 °C, whereas at 39 °C they differentiate into decidual cells. At 39 °C, the expression of *bax* was remarkably induced, whereas the increase in *bcl-*x_L expression was not as dramatic. No expression of *bcl-2* was observed. Also, the expression of *desmin* was increased at 39 °C, confirming that the cells decidualized at this temperature. (E) Ethidium bromide-stained 28S band. (F) The results were quantitated by densitometric analysis using rRNA to correct the intensities of mRNA expression.

Monitoring of apoptosis in GG-AD cells

Gu and colleagues have shown that apoptosis plays an important role during decidualization in animal models (3). In order to use this cell line as a model to investigate the molecular events during decidualization, we wanted to determine whether these cells undergo apoptosis at 39 °C. To monitor the apoptosis, we employed Annexin V staining as an indicator of apoptosis. This procedure is based on the observation that soon after the initiation of apoptosis, most cells translocate their phosphotidylserine from the plasma membrane to the cell surface (16). On the cell surface, phosphotidylserine can easily be detected by staining with FITC-conjugated Annexin (17, 18). GG-AD cells were grown at 33 °C (Fig. 2A, B) and at 39 °C (Fig. 2C, D). The cells show phosphotidylserine-positive apoptotic staining around their membranes at 39 °C (Fig. 2C) after culturing for 5 days. No staining was observed when cells were grown at 33 °C (Fig. 2A). For positive controls, we used 5% ethanol treatment for 90 min to induce early apoptotic signs at 33 °C and 39 °C (Fig. 2B and 2D respectively).

Growth characteristics of GG-AD cells

We reasoned that since GG-AD cells show early signs of apoptosis, the number of viable cells at 39 °C should decrease in due course. In order to test this premise, we plated 10^5 cells into 100 mm tissue culture dishes and maintained them at 33 °C and 39 °C. At different time intervals, cells were trypsinized and counted ($n = 3$) in a hematocytometer. As depicted in Fig. 3, cells at 33 °C showed an increased growth rate, whereas at 39 °C there was no increase in the

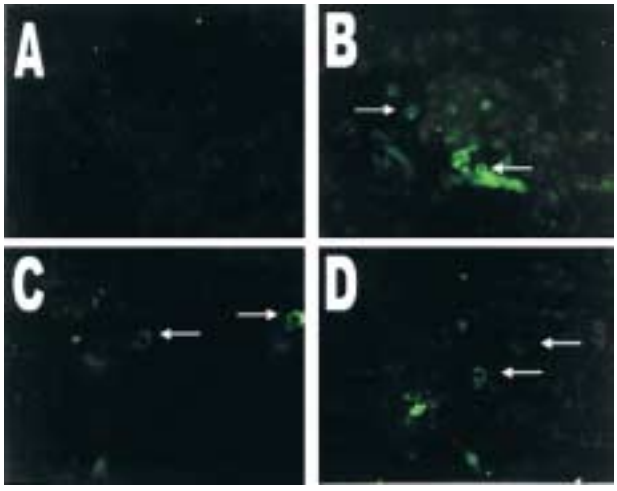

Figure 2 Detection of apoptosis with FITC-conjugated Annexin V in GG-AD cells at 33 °C (A) and 39 °C (C). GG-AD cells were also treated with 5% ethanol at 33 °C (B) and at 39 °C (D) to induce apoptosis and were used as positive controls. Arrows in (B), (C) and (D) show the Annexin V staining at the cell membranes. Observing the staining in (C) (39 °C) but not (A) (33 °C) suggested that cells at 39 °C showed the early signs of apoptosis (magnification: 40 ×).

number of cells. These data confirmed that a substantial number of GG-AD cells died at 39 °C. These results provide further support that the GG-AD cell line could be useful in assessing the relationship between apoptotic regulators and decidualization.

The expression of p53

During *in vivo* decidualization, when decidual cells undergo apoptosis, their p53 levels remain the same (19), suggesting that p53-independent pathways are responsible for the apoptosis. Therefore, we monitored the expression of p53 during *in vitro* decidualization. Protein extracts obtained from GG-AD cells at 33 °C and 39 °C and were subjected to Western blot analysis using p53 monoclonal antibody DO1. As shown in Fig. 4, our results indicated that p53 protein levels did not change, suggesting that apoptosis in GG-AD cells occurs by a p53-independent pathway similar to the *in vivo* decidualization, observed by Gibori *et al.* (19).

Discussion

Although there is diversity among species, the purpose of implantation is the same in all species: to attach the embryo to the uterus and to establish a close relationship between maternal and fetal tissues. Implantation failure is responsible for 22% of the spontaneous abortions that occur early in human pregnancy (20). In farm animals, 80% of embryonic loss occurs during the peri-implantation period (21). Thus, understanding the various signals and molecular pathways known to induce or regulate implantation is of great clinical as well as economic concern.

Use of an animal model for blastocyst implantation has allowed greater insight into the mechanisms

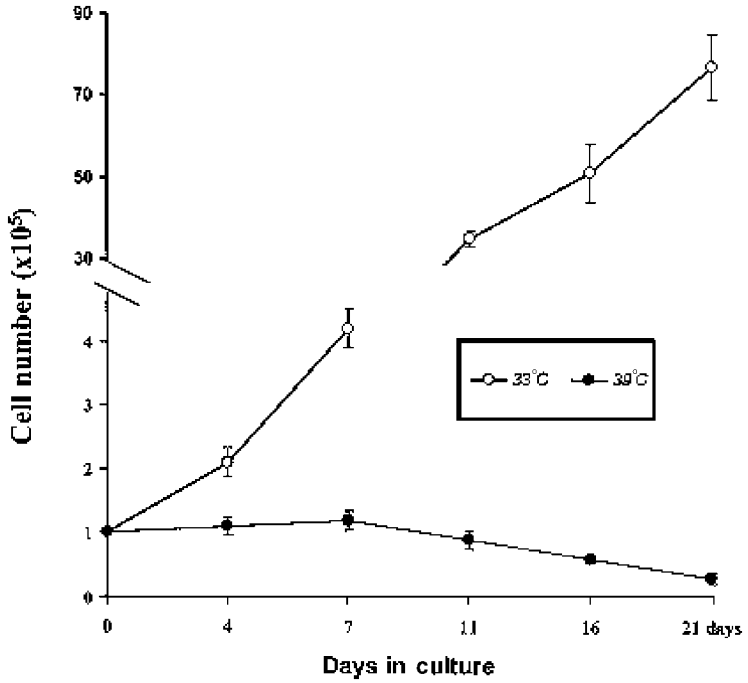

Figure 3 Growth curve of GG-AD cells that were grown at 33 °C (○) and 39 °C (●) ($n = 3$). Cells showed an increased growth rate at 33 °C but not at 39 °C.

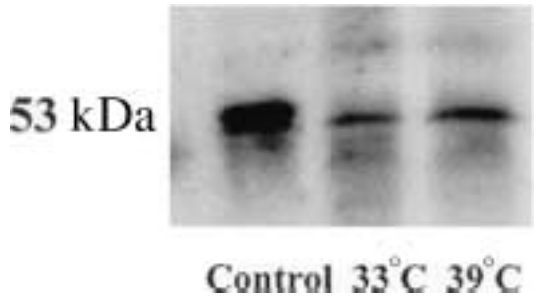

Figure 4 p53 expression during temperature-induced decidualization. The expression of p53 protein did not show any change at 33 °C and 39 °C. Protein extracted from u.v.-irradiated human melanocytes was used for the control group.

of implantation. However, due to the unavailability of a suitable decidual cell line, appropriate experiments could not be designed to probe the molecular mechanisms of blastocyst implantation. Cell lines were developed from endometrial uterine cells (22), but did not prove useful because these cells differed profoundly from decidual cells and failed to decidualize or express genes that encode decidual hormones (22). Attempts to use primary antimesometrial decidual cells also failed because of their inability to proliferate in primary cell culture. However a temperature-sensitive cell line (GG-AD) from the endocrine cells of rat decidua has been successfully established, circumventing many of the obstacles (9). These cells can be induced to undergo decidualization at the non-permissive temperature of 39 °C. Interestingly, when the temperature is shifted back to 33 °C, GG-AD cells dedifferentiate and regain features of the endometrial stromal cells. Several studies have already used this cell line to study the process of decidualization and observed similarities between this model and *in vivo* models of decidualization (10–12). Encouraged by these observations, we set out to examine the mechanism of apoptotic regression of decidual cells using the temperature-sensitive GG-AD cells as a model.

First we sought to explore whether this *in vitro* system is really a reflection of the *in vivo* animal model. Since we have shown that the expression of *bcl-2* family genes was altered during early pregnancy in our animal model (5), we decided to examine whether these genes were involved during *in vitro* decidualization. It has been proposed that the ratio of *bcl-2* to *bax* expression determines the fate of a cell, i.e. whether it should live or undergo programmed cell death (6–7). Also these three genes have been shown to form homo- or heterodimers in the mammalian system (23). Therefore, we chose to detect the expression level of *bax*, *bcl-2* and *bcl-*x_L amongst the *bcl-2* family of genes. Based on the Northern data for *bax*, *bcl-2* and *bcl-*x_L expression, this cell line seems to mimic what we observed previously during the decidualization of stromal cells *in vivo* (5). However, unlike the *in vivo* system, only the 1.0 kb transcript of *bax* was identified in this cell line. We did not detect the other alternatively spliced transcript, 1.5 kb *bax*β, in GG-AD cells. The functional difference between these two transcripts is not known but Bax protein is translated from the 1.0 kb transcript (*bax*α), whereas a protein corresponding to *bax*β has not been identified (S. Korsmeyer, personal communication). Since we used the whole uteri in our animal study, it is likely that *bax*β is contributed by the uterine cells, which are different from the parental GG-AD cells.

If the *in vitro* system of GG-AD cells is a true model for *in vivo* decidualization, then plausibly these cells must undergo apoptosis at the non-permissive temperature of 39 °C. Therefore, we wished to determine whether GG-AD cells show signs of apoptosis when they are grown at 39 °C. We explored this line of reasoning by measuring apoptosis in the GG-AD cells at 39 °C using the Annexin V staining method that has been optimized to detect the initial stages of apoptosis. It is believed that during apoptosis, phosphotidylserine is externalized prior to the known nuclear changes associated with apoptosis (24). Thus an assessment of the externalized phosphotidylserine by Annexin V provides a sensitive tool to measure early events in apoptosis (16–18). Our results with Annexin V indicated that, indeed, the GG-AD cells at 33 °C did not show any cell membrane staining, however at 39 °C, they showed the characteristic staining at their membrane after culturing 5 days, indicating that they underwent apoptosis at 39 °C. It is worth noting that the source of GG-AD cell line (antimesometrial decidual cells from day 10 of pseudopregnant rat) also showed signs of apoptosis as measured by DNA fragmentation. However, the extent of DNA fragmentation showed a marked increase at day 13/14 during *in vivo* decidualization (3). It is therefore likely that the antimesometrial decidual cells used by Srivastava *et al.* (9) in establishing the GG-AD cell line represent the decidual cells undergoing early stages of apoptosis. Our results using FITC-conjugated Annexin V staining support this contention.

Since the GG-AD cells show signs of early apoptosis at 39 °C, we reasoned that the number of viable cells at 39 °C should decrease during culture. In contrast, the GG-AD cells maintained at 33 °C should continue to divide and increase in number. Indeed, the GG-AD cells at 33 °C showed an increased growth rate, whereas at 39 °C there was no increase and the cells died 3 weeks after they were plated. Our observation that when maintained at 39 °C the number of GG-AD cells remained unchanged for the first 10 days would suggest that during this phase cell proliferation and cell death are occurring in parallel, resulting in a steady state. However, after 10 days the proportion of cells undergoing apoptosis takes over and results in the gradual loss of all cells at 39 °C by 3 weeks. Our data provide support for using the GG-AD cell line as a good model to explore the relationship between apoptotic regulators and decidual cell death.

Another similarity between *in vitro* and *in vivo* decidualization models is the status of p53 expression levels. p53 protein functions at least in part as a transcriptional regulator and can transactivate several cellular genes (25, 26). Two important events are regulated by p53 in connection with its function as a tumor suppressor. p53 has been shown to induce cell cycle arrest at the G1/S border, and to induce apoptosis (27, 28). Members of the *bcl-2* family genes, *bax* and *bcl-2* are known to be regulated by p53 (29). p53 activates *bax* (30), but inhibits the expression of *bcl-2* in certain cell lines (31). During *in vivo* and *in vitro* decidualization, it was expected that the expression of p53 would increase since in both model systems apoptotic cell death is evident. However, both during *in vivo* decidual cell regression (19) and during *in vitro* decidualization of GG-AD cells at 39 °C (this study), the p53 levels did not change. We collected these cells after 5 days of culture because this was the first day of detecting the early changes accompanied with apoptosis with our Annexin V staining. Based on this, we did not expect to see any changes in p53 expression at earlier days of GG-AD cell culture at 39 °C. These observations support a p53-independent pathway for the apoptosis of the decidual cells that is seen during early pregnancy. Though the *bax* gene promoter clearly has the potential to respond to p53 (30), it is also possible that other *cis*-acting elements within the *bax* promoter can mediate p53-independent transactivation of this gene or can modulate the influence of p53 on it. The promoter region of *bax* has been shown to have potential binding sites for different transcription factors, including Myc (32). Since c-Myc has been reported functionally to induce apoptosis (33), it may act as another transactivator of *bax*.

Other than transcriptional regulation, the Bax protein is also post-translationally modified (34–36). Recently, 16–18 kDa proteolytic fragments of the Bax protein were shown to promote apoptosis independent of p53 expression (34–36). It is probable that a 16–18 kDa proteolytic fragment of Bax promotes apoptosis in the decidual cells. Results presented in this study as well as those described previously by Gibori *et al.* (19) indicate that apoptotic events during placentation are independent of p53.

In conclusion, we have characterized the expression pattern of the *bcl-2* gene family in a T-antigen-transformed rat decidual cell line, GG-AD, at the permissive (33 °C) and non-permissive temperatures (39 °C). Our results established a relationship between the expression pattern of the *bcl-2* family of genes, and the onset of apoptosis with the decidualization and programmed cell death of GG-AD cells. Furthermore, our findings indicated that the programmed cell death of the decidual cells occurred in a p53-independent manner. Since these *in vitro* observations correlated well with the reported observations in animal models, the *in vitro* model of GG-AD cells could be used to study the mechanism of decidual cell regression.

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